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# Assembling the components of the quorum sensing pathway in African trypanosomes

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## Summary

African trypanosomes, parasites that cause human sleeping sickness, undergo a density-dependent differentiation in the bloodstream of their mammalian hosts. This process is driven by a released parasite-derived factor that causes parasites to accumulate in G1 and become quiescent. This is accompanied by morphological transformation to ‘stumpy’ forms that are adapted to survival and further development when taken up in the blood meal of tsetse flies, the vector for trypanosomiasis. Although the soluble signal driving differentiation to stumpy forms is unidentified, a recent genome-wide RNAi screen identified many of the intracellular signaling and effector molecules required for the response to this signal. These resemble components of nutritional starvation and quiescence pathways in other eukaryotes, suggesting that parasite development shares similarities with the adaptive quiescence of organisms such as yeasts and *Dictyostelium* in response to nutritional starvation

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1 and stress. Here, the trypanosome signaling pathway is discussed in the context of  
2 these conserved pathways and the possible contributions of opposing ‘slender  
3 retainer’ and ‘stumpy inducer’ arms described. As evolutionarily highly divergent  
4 eukaryotes, the organization and conservation of this developmental pathway can  
5 provide insight into the developmental cycle of other protozoan parasites, as well as  
6 the adaptive and programmed developmental responses of all eukaryotic cells.

## 7 8 9 **Introduction**

10 The ability of cells to sense their surroundings and respond to changes in the  
11 environment is fundamental to their survival. Monitoring cell density and its  
12 regulation is especially beneficial, ensuring the availability of nutrients and space and  
13 preventing the accumulation of toxic metabolic waste. This capacity of cells to sense  
14 their critical density, referred to as quorum sensing, requires signal molecule/s that  
15 serve as reporters of the population density and signal relay components that transmit  
16 this information within the cell, causing it to generate a response. The response to  
17 increased cell numbers is often a switch to quiescence (usually accompanied by  
18 morphogenetic and metabolic changes), a response that promotes survival of the cells  
19 under conditions of dwindling resources.

20  
21 African trypanosomes, the causative agents of sleeping sickness in humans and  
22 ‘nagana’ in livestock, are protozoan parasites that alternate their lives between a  
23 mammalian host and an insect vector, the tsetse fly. In the mammalian bloodstream,  
24 the parasites are ‘pleomorphic’ (i.e. of different morphologies) and undergo a  
25 developmental transition from rapidly proliferating long ‘slender’ forms to non-  
26 dividing short ‘stumpy’ forms, upon attaining a critical cell density (Fig. 1). The  
27 slender forms, if prevented from differentiation to stumpy forms, would result in the  
28 rapid death of the host through uncontrolled proliferation, as is the case with  
29 laboratory-adapted ‘monomorphic’ strains (Turner, 1990). Apart from the visible  
30 morphological changes, the differentiation to stumpy forms also entails several  
31 physiological changes in the parasite, which promote the survival of stumpy forms  
32 during uptake by the tsetse fly, and upon subsequent development to the procyclic  
33 forms in the insect midgut. The slender to stumpy switch is thus vital for controlling

the parasitaemia as well as for efficient transmission of the parasite to the fly, both of which aid in the successful maintenance and spread of infection.

#### The differentiation signal

Pleomorphs reportedly need to attain a threshold parasitaemia (apparently variable in different hosts) before differentiation and proliferation arrest in G1/G0 as stumpy forms can occur (Seed and Sechelski, 1989). Similarly, the cell cycle arrest of pleomorphic parasites grown *in vitro* is linked to their cell density, and this is irrespective of their initial seeding density (Reuner et al., 1997). These observations suggested the existence of a density sensing mechanism in trypanosomes and, further, the ability of plasma from infected animals at peak parasitaemia to inhibit trypanosome proliferation proposed the involvement of a secreted factor (Seed and Sechelski, 1989), although the accumulation of toxic inhibitors was not ruled out.

This scenario of trypanosomes undergoing growth arrest upon reaching a threshold density is resonant of Quorum Sensing (QS) pathways, widely described in bacteria and several lower eukaryotes. Gram-negative bacteria largely employ N-acyl-homoserine lactones (AHLs) as auto inducers of QS signaling cascades. The AHLs are freely diffusible molecules that can bind to and activate a transcriptional activator, which in turn induces expression of target genes. Another class of molecules that mediate similar effects is the 4-quinolones. On the other hand, Gram-positive bacteria use small post-translationally processed peptides as signal molecules. These peptides have been shown to interact with a histidine kinase two-component signal transduction system (Gonzalez and Keshavan, 2006, Atkinson and Williams, 2009, Deep et al., 2011). Beyond bacteria, several eukaryotes such as yeast and other fungal species communicate through diffusible molecules including farnesol and tyrosol and other small molecules (Hogan, 2006). Similarly, the social amoeba, *Dictyostelium discoideum*, in response to starvation, secretes an array of factors, including the glycoprotein conditioned medium factor (CMF). Additionally, the cells secrete pre-starvation factors (PSFs) during growth, which accumulate in the medium and hence serve as indicators of cell density. Other extracellular signals such as a chlorinated hydrocarbon called differentiation-inducing factor (DIF), the steroid, GABA and small peptides have also been shown to mediate QS in this slime mold (Clarke and Gomer, 1995, Gomer et al., 2011).

1  
2 In trypanosomes, while identification of the QS signaling molecule has evaded us to  
3 date, the evidence is that pleomorphs (and monomorphs) secrete a soluble, low  
4 molecular weight, heat stable factor/s termed Stumpy Induction Factor (SIF). SIF is of  
5 parasite origin and its accumulation in the culture medium/blood stream, with  
6 increasing parasite density, is proposed to act as the trigger for the slender to stumpy  
7 differentiation. This factor is believed to act on the same population that produces it,  
8 such that it induces an autocrine response. Monomorphs, in contrast, seem to be  
9 'signal-blind' to the factor they produce, thus providing them with a growth  
10 advantage *in vitro* (Vassella et al., 1997). This selective advantage is restricted to *in*  
11 *vitro* growth; *in vivo*, host longevity and the need for tsetse transmission would  
12 represent an important counter-selection to retain pleomorphism. This probably  
13 explains why monomorphs are not favoured in nature and reinforces the importance  
14 of density-dependent growth in the success of the trypanosomes' proficient parasitic  
15 lifestyle (MacGregor et al., 2012).

#### 16 17 **The SIF signaling pathway**

18 Lack of information on the chemical identity of SIF has made it difficult to  
19 understand the production of the signal, its turnover and the molecular cascade  
20 involved in relaying this signal. Early studies on slender forms noted a 2-3 fold  
21 increase in cyclic AMP (cAMP) levels at peak parasitaemia followed by a decline in  
22 its levels during the transition to stumpy forms (Mancini and Patton, 1981). Consistent  
23 with that, addition of conditioned medium containing SIF also resulted in a similar  
24 elevation in cAMP levels in pleomorphs, indicating a potential role for this secondary  
25 messenger in QS signaling (Vassella et al., 1997). A SIF-like growth inhibitory effect  
26 was also seen when pleomorphs were exposed to the cell permeable cAMP analogue  
27 (8-pCPT-cAMP) further supporting its possible role in this pathway (Vassella et al.,  
28 1997). Nevertheless, it was later shown that the active modulator was actually the  
29 hydrolysis product of 8-pCPT-cAMP, namely 8-pCPT-AMP (or its adenosine  
30 equivalent), since non-hydrolysable versions of 8-pCPT-cAMP were incapable of  
31 inducing growth arrest (Laxman et al., 2006). Consequently, this ruled out the  
32 participation of a canonical cAMP signaling mechanism in the differentiation process.  
33 Moreover, in contrast to their differential responsiveness to SIF, both pleomorphs and  
34 monomorphs responded to 8-pCPT-cAMP demonstrating that monomorphs, though

1 blind to SIF, still probably retained some of the components of the signaling pathway  
2 that drives stumpy formation(Laxman et al., 2006).

3  
4 This ability of monomorphs to respond to 8-pCPT-cAMP/AMP and undergo growth  
5 arrest has been exploited in a genome wide RNAi library screen that sought to select  
6 parasites that were unresponsive to 8-pCPT-cAMP/AMP after gene knockdown. The  
7 monomorphic library, capable of tetracycline induced gene silencing on a genome-  
8 wide scale, was exposed to 8-pCPT-cAMP/AMP, following which, growth was  
9 monitored and compared with the uninduced set. The parasites which failed to  
10 undergo growth arrest, were believed to continue proliferation due to the knock down  
11 of a gene required for sensing the 8-pCPT-cAMP/AMP signal. Deep sequencing of  
12 the RNAi inserts enriched in the selected 8-pCPT-cAMP/AMP resistant parasite  
13 population divulged a collection of genes representing various steps of a typical  
14 signaling pathway and likely to be involved in the 8-pCPT-cAMP/AMP response (Fig  
15 2). Crucially, when many of these genes were individually knocked down in  
16 pleomorphs by RNAi, they conferred resistance to SIF *in vivo* thereby confirming  
17 their involvement in the biologically relevant QS signaling pathway. This  
18 demonstrated that the 8-pCPT-cAMP/AMP mediated pathway intersected with the  
19 SIF signaling pathway at least to some extent, though inevitably molecules involved  
20 in the reception of the SIF signal were missing given the use of a cell permeable  
21 signal in the screen (Mony et al., 2013).

22  
23 This extensive screen thus gave the first insight into the complex machinery  
24 responsible for the slender to stumpy transition. The identified components ranged  
25 from characteristic cell signaling molecules such as phosphatases and kinases, to post  
26 transcriptional gene regulators such as RNA binding proteins. While arranging these  
27 components in precise order in the signaling cascade represents a significant  
28 experimental challenge, it is noteworthy that the list of genes identified overlap with  
29 those involved in nutritional stress induced dormancy of yeast and other lower  
30 eukaryotic cells (De Virgilio, 2012, Broach, 2012). It is tempting, therefore, to draw  
31 parallels with eukaryotic cell quiescence pathways to facilitate construction of a  
32 framework for the QS pathway in trypanosomes.

1 In the following sections we detail several of the protein families implicated in the  
2 control of stumpy formation and relate this to what is known in other eukaryotic  
3 organisms.

#### 4 5 A. Signaling molecules

##### 6 7 *Phosphatase cascades in trypanosome differentiation*

8 Protein phosphorylation plays a major role in the control and regulation of cellular  
9 functions in eukaryotic cells. This post translational modification occurs mainly on  
10 serine (Ser) or threonine (Thr) residues, making Ser/Thr protein phosphatases (PP)  
11 key players in the eukaryotic cell. The Ser/Thr protein phosphatases of type 1 (PP1)  
12 are the most abundant and among the best characterised protein phosphatase family.  
13 The PP1 catalytic subunit can interact with a large number of regulatory proteins,  
14 resulting in a conformational change of the phosphatase and a specific  
15 dephosphorylation of the target. Protein tyrosine phosphatases (PTPs), which also  
16 include dual-specificity phosphatases, dephosphorylate proteins at the tyrosine  
17 residues and play a major role in signal transduction. The ability of phosphatases to  
18 interact with multiple substrates explains their involvement in a wide range of cellular  
19 activities although there is limited information on their role in trypanosome biology.  
20 Nonetheless, a tyrosine phosphatase in *T. brucei*, *TbPTP1*, has previously been shown  
21 to play a key role in differentiation of the stumpy forms to procyclic forms (Szoor et  
22 al., 2006) with this acting on a serine threonine phosphatase that is targeted to  
23 glycosomes(Szoor et al., 2010). For the differentiation of slender parasites to stumpy  
24 forms, a PP1 and a dual specificity phosphatase (DsPhos) have been implicated  
25 experimentally(Mony et al., 2013).

26  
27 In yeast cells, PP1 (Glc7) plays a vital role in glucose signaling by interacting with a  
28 large number of regulatory proteins (e.g. Reg1). Glc7 is then capable of inactivating  
29 5'AMP activated protein kinase (AMPK) (Snf1 in yeast), which in turn transmits the  
30 glucose starvation signal (Sanz et al., 2000). Glc7 also dephosphorylates Msn2 and  
31 Msn4, the major stress-responsive transcription factors, causing their re-localization  
32 from the cytoplasm to the nucleus and hence activation of stress-responsive genes  
33 (Gorner et al., 1998, Gorner et al., 2002). In trypanosomes, members of a PP1 array  
34 (Tb927.4.3620-3640; PP1-3,4,5 (nomenclature based on (Li et al., 2006)) were shown

1 to be key inducers of stumpy formation, with their simultaneous RNAi-mediated  
2 knockdown preventing differentiation (Mony et al., 2013). Conversely, overexpression  
3 of one of these PP1 members drives cells in to G1 arrest (Mony and Matthews,  
4 unpublished observations). The coincident identification of an AMPK as a stumpy  
5 inducer, in the same RNAi screen, hints at the possible functioning of a similar  
6 mechanism in *T. brucei*. For example, one could speculate that PP1 acts upstream of  
7 AMPK in the SIF pathway such that AMPK depletion by RNAi might prevent the  
8 cell-cycle arrest generated upon PP1 overexpression, although PP1 might also have  
9 other targets in the pathway. Dual specificity phosphatases (DsPhos) in yeast have  
10 also been shown to regulate cell growth via regulation of the Protein kinase A (PKA)  
11 cascade (Beeser and Cooper, 2000) and could function analogously in trypanosomes,  
12 given that both a predicted DsPhos (Tb927.7.7160) and a PKA regulatory subunit  
13 (Tb927.11.4610) were identified in the screen for stumpy formation. Apart from these  
14 known functions, the phosphatases might also be operating through the deactivation  
15 of stumpy inhibitors or the slender ‘retainers’ (explained in a later section), molecules  
16 that would not have been identified in the genome-wide RNAi screen for drivers of  
17 stumpy formation.

#### 18 19 20 *Kinase mediated signaling of nutrient starvation*

21 A kinome-wide RNAi screen in *T. brucei* recently identified several novel kinases  
22 that functioned as cell cycle regulators as well as two kinases, RDK1 and RDK2, that  
23 apparently played a role in differentiation to procyclic forms (Jones et al., 2014). Two  
24 of the signaling cascades central to cellular quiescence in eukaryotes are those  
25 involving PKA and the Target of Rapamycin (TOR). The PKA pathway in yeast is  
26 largely responsible for the transcriptional changes associated with changes in glucose  
27 availability and leading to cellular quiescence (Zaman et al., 2008). The binding of  
28 cAMP to the regulatory subunit (PKA-R) alleviates the repression of the catalytic  
29 subunit (PKA-C), which in turn can now phosphorylate its substrates resulting in  
30 enhanced cellular proliferation. Thus, the increase in intracellular cAMP levels  
31 encountered during glucose rich conditions activate PKA-C and ensure continued cell  
32 proliferation. Conversely, glucose depletion is followed by a decrease in cAMP levels  
33 leaving PKA-R free to bind to PKA-C, leading to its inhibition and hence a G1  
34 arrest (Griffioen et al., 2000).



1  
2 Trypanosomes have a single gene encoding PKA-R and 3 orthologues for PKA-C,  
3 with the PKA-R gene (Tb927.11.4610) being identified in the RNAi screen for  
4 stumpy inducers. The RNAi mediated ablation of PKA-R (found in flagellar matrix)  
5 in monomorphs inhibited the motility of the parasites (Oberholzer et al., 2011), a  
6 phenomena thought to be regulated by cyclic nucleotide signaling as well. In  
7 *Dictyostelium discoideum*, disruption of PKA-R is known to keep PKA-C in a  
8 constitutively active state (Simon et al., 1992) such that in trypanosomes, the  
9 knockdown of PKA-R might keep PKA-C constitutively active and hence in a  
10 proliferative slender state. However, knockdown of PKA-R via RNAi in pleomorphs  
11 proved lethal for the parasite, probably due to a possible role in other cellular  
12 functions (as mentioned above) such that its rapid experimental validation for a role in  
13 stumpy induction was prevented (Mony et al., 2013).

14  
15 Parallel to the PKA pathway is the YAK kinase pathway that acts as a suppressor of  
16 proliferation (Garrett et al., 1991). In budding yeast, the YAK homologue, Yak1p has  
17 a function dependent on its cellular localization. Upon glucose starvation, it becomes  
18 translocated to the nucleus and more highly expressed, and is believed to  
19 phosphorylate PKA-R (Griffioen et al., 2001) along with other substrates.  
20 Phosphorylation of PKA-R in the nucleus results in its relocation to the cytoplasm,  
21 which probably results in inactivation of the PKA pathway and ultimately reduced  
22 cell proliferation (Zaman et al., 2008). The homologue of YAK in *Dictyostelium*,  
23 YAKA, induces a similar growth arrest (Souza et al., 1998) whereas in fission yeast, a  
24 related Dual specificity Yak Related Kinase (DYRK), Pom1p, generates a  
25 morphogenic gradient that prevents mitotic progression until cells reach an  
26 appropriate length (Bahler and Pringle, 1998, Aranda et al., 2011). The YAK kinase  
27 gene in *T. brucei* (Tb927.10.15020) has a potentially comparable function in cell  
28 cycle inhibition, since its depletion via RNAi results in prolonged cell proliferation  
29 and maintenance of slender forms (Mony et al., 2013) through as yet uncharacterized  
30 pathways. However, whether relocalisation to the nucleus contributes to its regulation  
31 of proliferation is unclear, given the focus of trypanosome gene regulation on post  
32 transcriptional rather than transcriptional events.

Members of the AMPK family (Snf in yeast and plants) act as major regulators of cellular energy homeostasis in eukaryotic cells. The AMPK cascade is regulated by the AMP:ATP ratio and hence acts as an energy sensor. A decline in ATP levels, along with a concomitant rise in AMP, activates this kinase, which then phosphorylates a range of downstream targets to bring about changes that reduce catabolic processes within the cell. AMPKs are thus capable of responding to a reduction in ATP levels (that may be brought about by nutrient depletion) by pushing the cell towards a state of reduced activity, in order to maintain the AMP:ATP ratio. Interestingly, AMPK is also known to be an inhibitor of the mTOR pathway (Carling et al., 2011). In trypanosomes, TOR4 was shown to be a negative regulator of stumpy induction (Barquilla et al., 2012), demonstrated by its knockdown in monomorphs, which drove the cells to develop features characteristic of stumpy forms. By this logic, one would thus expect that AMPK knockdown would maintain TOR4 in its active state and hence prevent differentiation, a prediction supported by the identification of an AMPK catalytic subunit in the RNAi screen for stumpy induction (Mony et al., 2013).

The Mitogen Activated Protein Kinase (MAPK) cascade in yeast is involved in stress responses as well as cell cycle regulation. This cascade, which involves a module of three protein kinases (MAPKKK, MAPKK, MAPK) that sequentially activate one another by phosphorylation, form a crucial part of intracellular signaling pathways in eukaryotic cells (Gustin et al., 1998). The most upstream kinase of this cascade is MAPKKK (MEK), and in trypanosomes, Tb927.2.2720 encodes a protein that contains the MEK domain. Knockdown of this gene renders pleomorphs non-responsive to SIF, suggesting a role for the MAPK pathway in QS signal transduction in trypanosomes (L. McDonald and K. R. Matthews, unpublished observations). Since in other eukaryotes, these kinases play a key role in conveying extracellular signals, it is likely that in trypanosomes, Tb927.2.2720 acts closely downstream of the unidentified SIF receptor, transmitting the external cell density signals to the cellular targets. This would thus place the MEK quite high up in the QS signalling cascade.

The NIMA (Never in Mitosis A) related kinases or NEKs, a class of serine/threonine kinases, were originally identified in a genetic screen for cell division cycle mutants in *Aspergillus nidulans*. The role of NEKs in cell cycle check point control has been

1 extensively reviewed in (Moniz et al., 2011, Fry et al., 2012) and may play a similar  
2 role in trypanosomes, where the NEK kinase family is considerably expanded (Jones  
3 et al., 2014). Members of this family are also implicated in quorum sensing since  
4 RNAi of one NEK family (Tb927.10.5930/5940/5950; these being indistinguishable  
5 by RNAi) delayed differentiation to stumpy forms, although this phenotype was less  
6 stringent than, for example, ablation of PP1 (where stumpy formation was completely  
7 abolished). This might reflect incomplete gene silencing or an incomplete penetrance  
8 of the genetic effect.

10 In summary, both protein phosphatases and protein kinases have been identified as  
11 regulators of the transition between slender and stumpy forms, though the targets  
12 upon which they operate are unknown. Nonetheless, phosphorylation is an expected  
13 contributor to the regulation of the signalling molecules themselves and also the  
14 effector molecules for differentiation upon which they may act, such as RNA binding  
15 proteins. Phosphoproteomic datasets analyzing cultured bloodstream and procyclic  
16 forms (Urbaniak et al., 2013, Nett et al., 2009) have already highlighted the potential  
17 for phosphorylation of many of the components identified from the screen for stumpy  
18 inducers; the consequences of these specific post translational modifications will  
19 require individual experimental analysis.

## 21 *B. Gene regulators*

23 The ultimate modulators in any signaling cascade are those that bring about gene  
24 expression and translational changes. Although the transcriptional silencer ISWI  
25 (Hughes et al., 2007, Stanne et al., 2011) was detected in the screen for stumpy  
26 inducers, more focus has been placed on post transcriptional regulation because  
27 several studies have found RNA binding proteins (RBPs) to play a key role in various  
28 stages of the trypanosome life cycle (Clayton, 2014, Kolev et al., 2014). While RBPs  
29 have been found to function in the bloodstream to procyclic transition and in  
30 metacyclogenesis (Kolev et al., 2012), RBP7 (representing two almost identical genes,  
31 RBP7A (Tb927.10.12090) and RBP7B (Tb927.10.12100)) is the sole candidate that  
32 has been experimentally demonstrated to be involved in the slender to stumpy  
33 differentiation. This protein has a single RNA recognition motif (RRM) and, while its  
34 overexpression prematurely drives parasites to stumpy forms, its knockdown results

1 in a delayed response to SIF. Analysis of the transcripts altered in abundance upon  
2 perturbed RBP7 expression (i.e. via RNAi or RBP7B overexpression) revealed, for  
3 example, the up regulation of several histone transcripts upon RBP7 knockdown,  
4 probably reflecting the continued proliferation of parasites as slender forms. In  
5 contrast, RBP7 overexpression increased the abundance of mRNAs of several other  
6 RNA binding proteins, but also certain procyclic form associated transcripts, which  
7 could reflect preparation for the next stage in the life cycle once stumpy cells are  
8 taken up by the tse-tse fly. The RRM motif found in RBP7 has sequence homology  
9 (~47%) to the RNA binding motif of the FCA protein in plants that is involved in the  
10 developmental transition to flowering (Macknight et al., 1997).

11  
12 As well as the proteins containing identifiable RNA binding motifs, a recent genome  
13 wide screen to identify posttranscriptional regulators in *T. brucei* (Erben et al., 2014)  
14 revealed three hypothetical proteins that had already been identified as regulators of  
15 stumpy formation (Mony et al., 2013) namely, Tb927.11.6600 (HYP 1),  
16 Tb927.9.4080 (HYP 2) and Tb927.11.2250 (HYP 12). Of these, HYP1 down-  
17 regulates mRNAs when artificially tethered to transcripts, whereas HYP2 and HYP11  
18 up-regulated bound mRNAs. HYP 1 and 2 were shown to be involved in stumpy  
19 induction through RNAi mediated knock down in pleomorphs (Mony et al., 2013) and  
20 possibly drive stumpy formation by destabilizing slender form ‘retainers’ or inhibitors  
21 of stumpy forms (for HYP1) or stabilizing drivers of stumpy formation (HYP2).  
22 HYP12, in contrast, has not yet been validated to have a role in stumpy formation  
23 through the creation of individual RNAi lines, but has nevertheless, been shown to  
24 bind RNA(Erben et al., 2014).

### 25 26 C. Hypothetical proteins

27  
28 Elucidating the exact role of a protein annotated as ‘hypothetical’ is a challenging task  
29 in trypanosome biology due to the lack of any information on its probable function.  
30 However, the fact that many hypothetical proteins identified in the screen are  
31 conserved across kinetoplastid species as well as the demonstration that two of them  
32 (HYP1 and HYP2) drive stumpy formation suggests they are fundamental  
33 components of a conserved cell communication or cell quiescence pathway found in  
34 other kinetoplastids. One observation from Table 1 (a list of hypothetical genes

driving stumpy formation) is that some of the hypothetical proteins harbour domains predicted to be involved in preprotein import into mitochondria (Tb927.4.3650 (HYP 4) and Tb927.9.13530 (HYP 6)) as well as there being a mitochondrial SSU subunit Tb927.11.11470 (HYP 13). This could mean that mitochondrial proteins contribute to stumpy formation and are not simply reflective of the mitochondrial elaboration that characterises stumpy formation. Also noteworthy is the identification of proteins with probable functions in ubiquitination (i.e. Tb927.8.2860, HYP 5; and Tb927.2.4020, a ubiquitin activating enzyme). These genes may be involved in the degradation of the slender cell proteins, which are no longer required in the next developmental stage or that act as repressors of stumpy formation. These predictions not only suggest that stumpy formation might be a multi-faceted process, but also highlight the importance of analysing these hypothetical proteins in uncovering aspects unique to kinetoplastid QS.

#### *D. Expression site components*

Pleomorphic parasites in the mammalian blood stream follow a characteristic undulating growth pattern, at least in experimental infections, with slender forms dominating until close to peak parasitaemia. These are replaced by transitional intermediate forms and then by the stumpy forms that predominate the declining phase till the next upsurge of slender forms sets in (Fig. 1). The slender forms that emerge in each new wave have an antigenically distinct coat of Variant Surface Glycoprotein (VSG), allowing them to proliferate in the face of host immunity to parasites generated in the first wave, such that antigenic waves and the slender to stumpy differentiation are temporally connected. However, recent evidence has highlighted the possibility of a mechanistic linkage also. The parasite harbours several hundred VSG genes clustered at subtelomeric regions, plus about 15-20 expression sites (ES) of which only a single expression site is active at any given time (Pays et al., 2001). Each of these ES also contains several other genes known as expression site-associated genes (ESAGs). While ES control and differentiation were believed to be mostly independent there is some evidence for co-ordination. Firstly, the slender to stumpy differentiation has been demonstrated to be accompanied by the silencing of the active ES (Amiguet-Vercher et al., 2004), though the actual molecular trigger is

not known. Also, VSG synthesis and cell cycle progression are closely linked since RNAi mediated knockdown of VSG resulted in a reversible cell cycle arrest, albeit at pre-cytokinesis rather than G1 (Sheader et al., 2005; Smith et al., 2009). Most recently, Batram *et al.* (Batram et al., 2014) have shown that ES attenuation, and more specifically depletion of three ESAGs (ESAG 1, 2 and 8), could trigger cell cycle arrest and the expression of the stumpy specific marker PAD1(Dean et al., 2009). However, unlike SIF mediated differentiation, this ES attenuation-mediated G1 arrest was reversible. It is thus possible that the G1 block observed after ES attenuation might be a consequence of the lack of the ESAG products. While ESAG1 function is poorly understood this protein is suggested to be localized to the cell surface (Cully et al., 1986, Pays et al., 2001). The function of ESAG2 is also unknown, but the presence of hydrophobic stretches in its encoded protein sequence indicate that this maybe a surface protein as well. ESAG 1 and 2 have also been localized to the flagellar pocket and could function as surface receptors (Pays et al., 2001). ESAG8, in contrast, has a nucleolar as well as cytoplasmic localization and interacts with a Pumilio domain containing protein, Puf1, denoting that it could act as a transcriptional or translational regulator(Hoek et al., 2002). In consequence, one could envision that these ESAGs are involved in the proliferative physiology of the slender forms such that their depletion might generate a nutritional starvation or stress-like environment that invokes a stumpy formation response through a signaling pathway that intersects with components of the SIF pathway. Although this could be beneficial to the cell by ensuring that successful ES switching is achieved (accompanied by ESAG1, 2 and 8 re-expression) and proliferation can recommence, the frequency of switching in the bloodstream parasite population would render this a minor contributor to the overall development of arrested, transmissible, stages in the blood.

## **Integrating the known networks**

While the discovery of various components of the stumpy induction pathway by the genome-wide screen has given a substantially better understanding of how trypanosomes positively drive stumpy formation, earlier studies had already identified possible negative regulators of the process. For example, the ablation of a Zinc finger kinase (ZFK) in pleomorphs inhibited growth and also increased the rate of slender to stumpy transition *in vitro*, though not *in vivo* (Vassella et al., 2001). Similarly, the creation of a null mutant for a mitogen activated protein kinase homologue,

1 TbMAPK5, gave a similar phenotype with the exception that the phenotype was also  
2 seen *in vivo*, thus reducing the chronicity of mouse infections (Domenicali Pfister et  
3 al., 2006). While the ZFK and MAPK knockouts could induce differentiation only in  
4 pleomorphs, RNAi of the TOR kinase, TbTOR4 in monomorphs triggered a transition  
5 to the stumpy form(Barquilla et al., 2012), as highlighted earlier. This may reflect the  
6 existence of redundant signaling pathways or a defect in the pathway in monomorphs  
7 at a point downstream of ZFK and MAPK, but upstream of TbTOR4.

8  
9 Combined, one can speculate that trypanosomes have a dual mechanism for  
10 controlling differentiation, comprising of a slender retention (SR) as well a stumpy  
11 induction (SI) arm (Fig 3). A set of genes, the 'slender retainers' (ZFK, MAPK5,  
12 TOR4 etc.) is constitutively expressed and their products keep the cell in an actively  
13 proliferating state. This slender state is retained until SIF accumulation reaches  
14 threshold levels, triggering the 'nutritional stress -like' response, which leads to  
15 concomitant repression of the 'slender retainers' and activation of 'stumpy inducers'.  
16 This tip in the equilibrium towards stumpy induction, apart from causing cell cycle  
17 arrest, also starts preparing the cell for its next life cycle stage in the insect by re-  
18 activating mitochondrial functions required for oxidative phosphorylation as well as  
19 by removing the proteins that were once needed for the maintenance of slender forms  
20 (for example by ubiquitination). Thus, the transformation to stumpy forms would be  
21 rendered irreversible through the coupled inactivation of the SRs and activation of  
22 SIs. Hence, it is the fine control of these two processes that may determine the  
23 balance of proliferation vs. differentiation, though the relative dominance of slender  
24 retention versus stumpy induction components remains to be determined, as does the  
25 point of commitment in this transition. Another aspect worth noting is the possibility  
26 of redundancy in the QS sensing pathway, as seen in the case of the response to  
27 nutritional starvation in yeast (Granek et al., 2011).

28  
29 A less investigated, but crucial aspect of the differentiation process is the timing of  
30 the onset of the commitment to differentiation from the long, slender to the short,  
31 stumpy form. While asymmetric division was suggested to be the means by which  
32 trypanosomes underwent commitment and morphological transformation (Tyler et al.,  
33 2001, Sharma et al., 2008), it would be difficult, with currently available data, to map  
34 out the exact timeline of events comprising the response to SIF and the subsequent

1 cell cycle arrest, although the subsequent organelle reorganization and changes in cell  
2 shape have been described(Vanhollebeke et al., 2010, MacGregor et al., 2012).  
3 However, one could speculate that SIF induced downregulation of SRs could be  
4 augmented by the dilution of these factors during the division of slender cells  
5 undergoing commitment to differentiation but that have not yet undergone  
6 arrest(Macgregor et al., 2011). Any asymmetry in these divisions (and the distribution  
7 of SRs) could determine whether one or both daughters ultimately commits to arrest  
8 and differentiation.

10 One piece of experimental evidence that supports a tension between stumpy inhibition  
11 and induction is the observation that, despite effective mRNA and protein elevation,  
12 the overexpression of ectopic RBP7B does not drive complete stumpy  
13 formation(Mony et al., 2013). Although other reasons could contribute to inefficient  
14 differentiation (such as the requirement for an interacting protein or a post  
15 translational modification of RBP7 for it to be completely active), this phenotype  
16 could also be due to the continued presence of ‘slender retainers’. Also possible is the  
17 presence of multiple or hierarchical ‘stumpy inducing’ arms in the pathway such that  
18 cell cycle arrest might precede (and be dissociable from) irreversible commitment and  
19 morphological transformation to stumpy forms, a concept supported by the observed  
20 effect of ESAG depletion on reversible G1 arrest and the fact that AMPK and TOR  
21 kinase have antagonizing consequences (see above) on stumpy formation. Clearly, it  
22 would be enormously valuable to identify slender retainers to facilitate a better  
23 understanding of the molecules that oppose the action of the stumpy inducers  
24 identified in the genome-wide RNAi screen and the QS pathway. The availability of  
25 an over-expression library (Erben et al., 2014) now makes it feasible to screen for  
26 such negative regulators of stumpy formation.

### 28 **QS interference, interspecies interactions and communication with the host**

29 Studies on QS in prokaryotes have revealed that the communication between cells can  
30 be far more complex than a simple autocrine effect. Apart from communicating with  
31 themselves, bacteria have been shown to intercept the signals of others in a niche  
32 containing several species (Deng et al., 2014b). Numerous eukaryotes have also been  
33 shown to be capable of perceiving prokaryotic QS signals (Atkinson and Williams,  
34 2009, Deng et al., 2014a). Although such information on QS in trypanosomes is



currently lacking, it represents an area of interest because trypanosomes not only interact within and with the host but also exist in the mammalian bloodstream in the company of other parasites. Infections with multiple species of trypanosomes are common in sub Saharan Africa(Cox et al., 2010), and there are currently uncharacterized possible interactions between them, involving either competition or co-operation. Moreover, the difficulty in producing biochemically and morphologically replete stumpy forms *in vitro* also suggests that the host bloodstream environment could contribute additional physical or biological factors in the process. Understanding these *in vivo* contributors will be important in dissecting the stumpy formation pathway and its impact, in different hosts, on parasite virulence and transmission. Of course, a detailed knowledge of the molecular components of the SIF pathway could also prove useful in the development of drugs that interfere with or mimic parasite communication, thus perturbing parasite virulence and transmission potential.

## Conclusions

With the development of tools such as the genome wide RNAi (Alsford et al., 2011) and overexpression (Erben et al., 2014) libraries, it has now become possible to carry out selective screens in *T. brucei* to identify the molecular components of biological pathways in addition to simple drug resistance mechanisms. One such screen, using a chemical inducer (8-cPT-cAMP/AMP) of stumpy-like forms *in vitro* has led to the discovery of various components of the SIF pathway(Mony et al., 2013). While this review has attempted to assemble those components based on *in silico* information, the existing literature as well as experimental data from other eukaryotes (Fig 4), validation of these hypotheses through experimentation is a significant challenge, especially where pathway components have other roles in the cell's physiology. Indeed, the identified pathway components are very likely to be part of an incomplete list, since stumpy inducers that are also essential proteins in the parasite would not have become enriched in the screen. It is thus crucial to dissect and further add to this pathway through multiple approaches. As an example, a recent high throughput chemical screen identified a novel chemical inducer of stumpy forms (MacGregor et al., 2014) allowing a chemical-genetic dissection of the pathway. Phosphoproteomic and transcriptomic approaches will also help in the identification of potential targets of the known components of the SIF pathway, especially of the phosphatases and

kinases. Nonetheless, although a great deal of further effort is needed to characterise this pathway in detail, it already represents the most comprehensively characterized signaling pathway in any eukaryotic parasite. Hence, discoveries related to trypanosome development could inform similar cell-cell communication pathways in other parasites such as that regulating gametocytogenesis in *Plasmodium* (Ikadai et al., 2013) as well as the regulation of developmental responses of other kinetoplastids, including *T. cruzi* and *Leishmania spp.*. The evolutionarily ancient position of trypanosomes in the eukaryotic lineage also has the potential to inform understanding of the molecular basis of cellular quiescence in other cells including those of mammals, whose breakdown is a key contributor to the development of many cancers.

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Gene Id	Name	Domains	Predicted function
Tb927.11.6600	Hyp 1	No conserved domain	Down regulates artificially tethered transcripts (Erben et al., 2014)
Tb927.9.4080	Hyp 2	Truncated AAA+ and Adenylation domains	ATP-dependent polynucleotide ligase; Up regulates artificially tethered transcripts (Erben et al., 2014)
Tb927.4.670	Hyp 3	GAF domain	Small molecule (cyclic nucleotide) binding, protein-protein interactions; Found in phosphodiesterases (Heikaus et al., 2009)
Tb927.4.3650 <sup>a</sup>	Hyp 4	No conserved domains	May function as a mitochondrial import receptor subunit TOM6 homolog (34%) involved in translocation of preproteins across mitochondrial outer membranes (Dukanovic et al., 2009)
Tb927.8.2860	Hyp 5	Truncated Mod(r) domain	Endosomal protein sortin; recognition of monoubiquitinated cargo proteins, mainly surface proteins such as transporters and receptors (Winter and Hauser, 2006)
Tb927.9.13530	Hyp 6	Pam16	Preprotein import into the mitochondrial matrix (Frazier et al., 2004)
Tb927.10.12110 <sup>a,b</sup>	Hyp 7	No conserved domain	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 1 (48%)
Tb927.11.300	Hyp 8	Truncated Bud13 domain	Pre-mRNA splicing and retention
Tb927.11.750	Hyp 9	Domains of Helicase, RING-finger, zf-PARP ; disrupted domain of DEAD-like helicase	Transcription / DNA replication, recombination, and repair; RING-finger: DNA binding, cell signaling, ubiquitination (Tuo et al., 2012)
Tb927.11.1640	Hyp 10	No conserved domains	None
Tb927.11.2250	Hyp 11	Truncated domain of Spumavirus Gag protein	Genome packaging, virion assembly, trafficking and membrane targeting in foamy viruses (Goldstone et al., 2013); Upregulates artificially tethered transcripts (Erben et al., 2014)
Tb927.11.6610 <sup>b</sup>	Hyp 12	Truncated RRM domain	Regulation of post transcriptional gene expression
Tb927.11.11470	Hyp 13	No conserved domains	Putative Mitochondrial SSU ribosomal protein

1 **Table 1**

2 *Genes identified in the genome wide RNAi screen for drivers of stumpy formation.*

3 **Notes:**

4 <sup>a</sup> Function prediction based on overall sequence homology rather than the presence of  
5 a conserved domain, with % identity shown in parentheses.

6 <sup>b</sup> This gene has yet to be confirmed with an independent RNAi line  
7  
8

## References

- ALSFORD, S., TURNER, D. J., OBADO, S. O., SANCHEZ-FLORES, A., GLOVER, L., BERRIMAN, M., HERTZ-FOWLER, C. & HORN, D. 2011. High-throughput phenotyping using parallel sequencing of RNA interference targets in the African trypanosome. *Genome Res.*
- ARANDA, S., LAGUNA, A. & DE LA LUNA, S. 2011. DYRK family of protein kinases: evolutionary relationships, biochemical properties, and functional roles. *FASEB J*, 25, 449-62.
- ATKINSON, S. & WILLIAMS, P. 2009. Quorum sensing and social networking in the microbial world. *Journal of the Royal Society, Interface / the Royal Society*, 6, 959-78.
- BAHLER, J. & PRINGLE, J. R. 1998. Pom1p, a fission yeast protein kinase that provides positional information for both polarized growth and cytokinesis. *Genes Dev*, 12, 1356-70.
- BARQUILLA, A., SALDIVIA, M., DIAZ, R., BART, J. M., VIDAL, I., CALVO, E., HALL, M. N. & NAVARRO, M. 2012. Third target of rapamycin complex negatively regulates development of quiescence in *Trypanosoma brucei*. *Proc Natl Acad Sci U S A*, 109, 14399-404.
- BATRAM, C., JONES, N. G., JANZEN, C. J., MARKERT, S. M. & ENGSTLER, M. 2014. Expression site attenuation mechanistically links antigenic variation and development in *Trypanosoma brucei*. *Elife*, 3, e02324.
- BEESE, A. E. & COOPER, T. G. 2000. The dual-specificity protein phosphatase Yvh1p regulates sporulation, growth, and glycogen accumulation independently of catalytic activity in *Saccharomyces cerevisiae* via the cyclic AMP-dependent protein kinase cascade. *Journal of bacteriology*, 182, 3517-28.
- BROACH, J. R. 2012. Nutritional control of growth and development in yeast. *Genetics*, 192, 73-105.
- CARLING, D., MAYER, F. V., SANDERS, M. J. & GAMBLIN, S. J. 2011. AMP-activated protein kinase: nature's energy sensor. *Nature chemical biology*, 7, 512-8.
- CLARKE, M. & GOMER, R. H. 1995. PSF and CMF, autocrine factors that regulate gene expression during growth and early development of *Dictyostelium*. *Experientia*, 51, 1124-1134.
- CLAYTON, C. E. 2014. Networks of gene expression regulation in *Trypanosoma brucei*. *Mol Biochem Parasitol*.
- COX, A. P., TOSAS, O., TILLEY, A., PICOZZI, K., COLEMAN, P., HIDE, G. & WELBURN, S. C. 2010. Constraints to estimating the prevalence of trypanosome infections in East African zebu cattle. *Parasit Vectors*, 3, 82.
- CULLY, D. F., GIBBS, C. P. & CROSS, G. A. 1986. Identification of proteins encoded by variant surface glycoprotein expression site-associated genes in *Trypanosoma brucei*. *Mol Biochem Parasitol*, 21, 189-97.
- DE VIRGILIO, C. 2012. The essence of yeast quiescence. *Fems Microbiology Reviews*, 36, 306-339.
- DEAN, S., MARCHETTI, R., KIRK, K. & MATTHEWS, K. R. 2009. A surface transporter family conveys the trypanosome differentiation signal. *Nature*, 459, 213-7.

- 1 DEEP, A., CHAUDHARY, U. & GUPTA, V. 2011. Quorum sensing and Bacterial  
2 Pathogenicity: From Molecules to Disease. *Journal of laboratory*  
3 *physicians*, 3, 4-11.
- 4 DENG, Y., LIM, A., LEE, J., CHEN, S., AN, S., DONG, Y. H. & ZHANG, L. H. 2014a.  
5 Diffusible signal factor (DSF) quorum sensing signal and structurally  
6 related molecules enhance the antimicrobial efficacy of antibiotics against  
7 some bacterial pathogens. *BMC microbiology*, 14, 51.
- 8 DENG, Y., LIM, A., LEE, J., CHEN, S., AN, S., DONG, Y. H. & ZHANG, L. H. 2014b.  
9 Diffusible signal factor (DSF) quorum sensing signal and structurally  
10 related molecules enhance the antimicrobial efficacy of antibiotics against  
11 some bacterial pathogens. *BMC Microbiol*, 14, 51.
- 12 DOMENICALI PFISTER, D., BURKARD, G., MORAND, S., RENGGLI, C. K., RODITI, I.  
13 & VASSELLA, E. 2006. A Mitogen-activated protein kinase controls  
14 differentiation of bloodstream forms of *Trypanosoma brucei*. *Eukaryot*  
15 *Cell*, 5, 1126-35.
- 16 DUKANOVIC, J., DIMMER, K. S., BONNEFOY, N., KRUMPE, K. & RAPAPORT, D.  
17 2009. Genetic and Functional Interactions between the Mitochondrial  
18 Outer Membrane Proteins Tom6 and Sam37. *Molecular and Cellular*  
19 *Biology*, 29, 5975-5988.
- 20 ERBEN, E. D., FADDA, A., LUEONG, S., HOHEISEL, J. D. & CLAYTON, C. 2014. A  
21 genome-wide tethering screen reveals novel potential post-  
22 transcriptional regulators in *Trypanosoma brucei*. *PLoS Pathog*, 10,  
23 e1004178.
- 24 FRAZIER, A. E., DUDEK, J., GUIARD, B., VOOS, W., LI, Y. F., LIND, M., MEISINGER, C.,  
25 GEISLER, A., SICKMANN, A., MEYER, H. E., BILANCHONE, V., CUMSKY, M.  
26 G., TRUSCOTT, K. N., PFANNER, N. & REHLING, P. 2004. Pam16 has an  
27 essential role in the mitochondrial protein import motor. *Nature*  
28 *Structural & Molecular Biology*, 11, 226-233.
- 29 FRY, A. M., O'REGAN, L., SABIR, S. R. & BAYLISS, R. 2012. Cell cycle regulation by  
30 the NEK family of protein kinases. *Journal of Cell Science*, 125, 4423-4433.
- 31 GARRETT, S., MENOLD, M. M. & BROACH, J. R. 1991. The *Saccharomyces*  
32 *cerevisiae* YAK1 gene encodes a protein kinase that is induced by arrest  
33 early in the cell cycle. *Mol Cell Biol*, 11, 4045-52.
- 34 GOLDSTONE, D. C., FLOWER, T. G., BALL, N. J., SANZ-RAMOS, M., YAP, M. W.,  
35 OGRODOWICZ, R. W., STANKE, N., REH, J., LINDEMANN, D., STOYE, J. P. &  
36 TAYLOR, I. A. 2013. A Unique Spumavirus Gag N-terminal Domain with  
37 Functional Properties of Orthoretroviral Matrix and Capsid. *PLoS*  
38 *pathogens*, 9.
- 39 GOMER, R. H., JANG, W. & BRAZILL, D. 2011. Cell density sensing and size  
40 determination. *Development Growth & Differentiation*, 53, 482-494.
- 41 GONZALEZ, J. E. & KESHAVAN, N. D. 2006. Messing with bacterial quorum  
42 sensing. *Microbiology and Molecular Biology Reviews*, 70, 859-+.
- 43 GORNER, W., DURCHSCHLAG, E., MARTINEZ-PASTOR, M. T., ESTRUCH, F.,  
44 AMMERER, G., HAMILTON, B., RUIS, H. & SCHULLER, C. 1998. Nuclear  
45 localization of the C2H2 zinc finger protein Msn2p is regulated by stress  
46 and protein kinase A activity. *Genes Dev*, 12, 586-97.
- 47 GORNER, W., DURCHSCHLAG, E., WOLF, J., BROWN, E. L., AMMERER, G., RUIS, H.  
48 & SCHULLER, C. 2002. Acute glucose starvation activates the nuclear

- 1 localization signal of a stress-specific yeast transcription factor. *EMBO J*,  
2 21, 135-44.
- 3 GRANEK, J. A., KAYIKCI, O. & MAGWENE, P. M. 2011. Pleiotropic signaling  
4 pathways orchestrate yeast development. *Current Opinion in*  
5 *Microbiology*, 14, 676-681.
- 6 GRIFFIOEN, G., ANGHILERI, P., IMRE, E., BARONI, M. D. & RUIS, H. 2000.  
7 Nutritional control of nucleocytoplasmic localization of cAMP-dependent  
8 protein kinase catalytic and regulatory subunits in *Saccharomyces*  
9 *cerevisiae*. *Journal of Biological Chemistry*, 275, 1449-1456.
- 10 GRIFFIOEN, G., BRANDUARDI, P., BALLARINI, A., ANGHILERI, P., NORBECK, J.,  
11 BARONI, M. D. & RUIS, H. 2001. Nucleocytoplasmic distribution of  
12 budding yeast protein kinase A regulatory subunit Bcy1 requires Zds1  
13 and is regulated by Yak1-dependent phosphorylation of its targeting  
14 domain. *Molecular and Cellular Biology*, 21, 511-523.
- 15 GUSTIN, M. C., ALBERTYN, J., ALEXANDER, M. & DAVENPORT, K. 1998. MAP  
16 kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiology and*  
17 *Molecular Biology Reviews*, 62, 1264-+.
- 18 HEIKAUS, C. C., PANDIT, J. & KLEVIT, R. E. 2009. Cyclic Nucleotide Binding GAF  
19 Domains from Phosphodiesterases: Structural and Mechanistic Insights.  
20 *Structure*, 17, 1551-1557.
- 21 HOEK, M., ZANDERS, T. & CROSS, G. A. 2002. Trypanosoma brucei expression-  
22 site-associated-gene-8 protein interacts with a Pumilio family protein.  
23 *Mol Biochem Parasitol*, 120, 269-83.
- 24 HOGAN, D. A. 2006. Talking to themselves: Autoregulation and quorum sensing  
25 in fungi. *Eukaryotic cell*, 5, 613-619.
- 26 HUGHES, K., WAND, M., FOULSTON, L., YOUNG, R., HARLEY, K., TERRY, S.,  
27 ERSFELD, K. & RUDENKO, G. 2007. A novel ISWI is involved in VSG  
28 expression site downregulation in African trypanosomes. *EMBO J*, 26,  
29 2400-10.
- 30 IKADAI, H., SALIBA, K. S., KANZOK, S. M., MCLEAN, K. J., TANAKA, T. Q., CAO, J.,  
31 WILLIAMSON, K. C. & JACOBS-LORENA, M. 2013. Transposon mutagenesis  
32 identifies genes essential for Plasmodium falciparum gametocytogenesis.  
33 *Proceedings of the National Academy of Sciences of the United States of*  
34 *America*, 110, E1676-E1684.
- 35 JONES, N. G., THOMAS, E. B., BROWN, E., DICKENS, N. J., HAMMARTON, T. C. &  
36 MOTTRAM, J. C. 2014. Regulators of Trypanosoma brucei cell cycle  
37 progression and differentiation identified using a kinome-wide RNAi  
38 screen. *PLoS Pathog*, 10, e1003886.
- 39 KOLEV, N. G., RAMEY-BUTLER, K., CROSS, G. A., ULLU, E. & TSCHUDI, C. 2012.  
40 Developmental progression to infectivity in Trypanosoma brucei  
41 triggered by an RNA-binding protein. *Science*, 338, 1352-3.
- 42 KOLEV, N. G., ULLU, E. & TSCHUDI, C. 2014. The emerging role of RNA-binding  
43 proteins in the life cycle of Trypanosoma brucei. *Cell Microbiol*, 16, 482-9.
- 44 LAXMAN, S., RIECHERS, A., SADILEK, M., SCHWEDE, F. & BEAVO, J. A. 2006.  
45 Hydrolysis products of cAMP analogs cause transformation of  
46 Trypanosoma brucei from slender to stumpy-like forms. *Proc Natl Acad*  
47 *Sci USA*, 103, 19194-9.

- 1 LI, Z., TU, X. & WANG, C. C. 2006. Okadaic acid overcomes the blocked cell cycle  
2 caused by depleting Cdc2-related kinases in *Trypanosoma brucei*. *Exp Cell*  
3 *Res*, 312, 3504-16.
- 4 MACGREGOR, P., IVENS, A., SHAVE, S., COLLIE, I., GRAY, D., AUER, M. &  
5 MATTHEWS, K. R. 2014. High-throughput chemical screening for  
6 antivirulence developmental phenotypes in *Trypanosoma brucei*.  
7 *Eukaryot Cell*, 13, 412-26.
- 8 MACGREGOR, P., SAVILL, N. J., HALL, D. & MATTHEWS, K. R. 2011. Transmission  
9 Stages Dominate Trypanosome Within-Host Dynamics during Chronic  
10 Infections. *Cell Host Microbe*, 9, 310-8.
- 11 MACGREGOR, P., SZOOR, B., SAVILL, N. J. & MATTHEWS, K. R. 2012.  
12 Trypanosomal immune evasion, chronicity and transmission: an elegant  
13 balancing act. *Nat Rev Microbiol*, 10, 431-8.
- 14 MACKNIGHT, R., BANCROFT, I., PAGE, T., LISTER, C., SCHMIDT, R., LOVE, K.,  
15 WESTPHAL, L., MURPHY, G., SHERSON, S., COBBETT, C. & DEAN, C. 1997.  
16 FCA, a gene controlling flowering time in *Arabidopsis*, encodes a protein  
17 containing RNA-binding domains. *Cell*, 89, 737-45.
- 18 MANCINI, P. E. & PATTON, C. L. 1981. Cyclic 3',5'-adenosine monophosphate  
19 levels during the developmental cycle of *Trypanosoma brucei brucei* in  
20 the rat. *Mol Biochem Parasitol*, 3, 19-31.
- 21 MONIZ, L., DUTT, P., HAIDER, N. & STAMBOLIC, V. 2011. Nek family of kinases in  
22 cell cycle, checkpoint control and cancer. *Cell Division*, 6.
- 23 MONY, B., MACGREGOR, P., IVENS, A., COWTON, A., WILSON, J., HORN, D. &  
24 MATTHEWS, K. 2013. Genome wide dissection of the quorum sensing  
25 signaling pathway in *Trypanosoma brucei*. *Nature*,  
26 10.1038/nature12864.
- 27 NETT, I. R., MARTIN, D. M., MIRANDA-SAAVEDRA, D., LAMONT, D., BARBER, J. D.,  
28 MEHLERT, A. & FERGUSON, M. A. 2009. The phosphoproteome of  
29 bloodstream form *Trypanosoma brucei*, causative agent of African  
30 sleeping sickness. *Mol Cell Proteomics*, 8, 1527-38.
- 31 PAYS, E., LIPS, S., NOLAN, D., VANHAMME, L. & PEREZ-MORGA, D. 2001. The VSG  
32 expression sites of *Trypanosoma brucei*: multipurpose tools for the  
33 adaptation of the parasite to mammalian hosts. *Mol Biochem Parasitol*,  
34 114, 1-16.
- 35 REUNER, B., VASSELLA, E., YUTZY, B. & BOSHART, M. 1997. Cell density triggers  
36 slender to stumpy differentiation of *Trypanosoma brucei* bloodstream  
37 forms in culture. *Mol Biochem Parasitol*, 90, 269-80.
- 38 SANZ, P., ALMS, G. R., HAYSTEAD, T. A. & CARLSON, M. 2000. Regulatory  
39 interactions between the Reg1-Glc7 protein phosphatase and the Snf1  
40 protein kinase. *Mol Cell Biol*, 20, 1321-8.
- 41 SEED, J. R. & SECHELSKI, J. B. 1989. Mechanism of long slender (LS) to short  
42 stumpy (SS) transformation in the African trypanosomes. *J Protozool*, 36,  
43 572-7.
- 44 SIMON, M. N., PELEGRINI, O., VERON, M. & KAY, R. R. 1992. Mutation of Protein  
45 Kinase-a Causes Heterochronic Development of *Dictyostelium*. *Nature*,  
46 356, 171-172.
- 47 SOUZA, G. M., LU, S. J. & KUSPA, A. 1998. YakA, a protein kinase required for the  
48 transition from growth to development in *Dictyostelium*. *Development*,  
49 125, 2291-2302.



- 1 STANNE, T. M., KUSHWAHA, M., WAND, M., TAYLOR, J. E. & RUDENKO, G. 2011.  
2 TbISWI regulates multiple polymerase I (Pol I)-transcribed loci and is  
3 present at Pol II transcription boundaries in *Trypanosoma brucei*.  
4 *Eukaryot Cell*, 10, 964-76.
- 5 SZOOR, B., RUBERTO, I., BURCHMORE, R. & MATTHEWS, K. 2010. A novel  
6 phosphatase cascade regulates differentiation in trypanosomes via a  
7 glycosomal signaling pathway. *Genes and Development*, 24, 1306-1316.
- 8 SZOOR, B., WILSON, J., MCELHINNEY, H., TABERNERO, L. & MATTHEWS, K. R.  
9 2006. Protein tyrosine phosphatase TbPTP1: a molecular switch  
10 controlling life cycle differentiation in trypanosomes. *J Cell Biol*, 175, 293-  
11 303.
- 12 TUO, S., NAKASHIMA, K. & PRINGLE, J. R. 2012. Apparent Defect in Yeast Bud-Site  
13 Selection Due to a Specific Failure to Splice the Pre-mRNA of a Regulator  
14 of Cell-Type-Specific Transcription. *Plos One*, 7.
- 15 TURNER, C. M. 1990. The use of experimental artefacts in African trypanosome  
16 research. *Parasitol Today*, 6, 14-7.
- 17 URBANIAK, M. D., MARTIN, D. M. & FERGUSON, M. A. 2013. Global quantitative  
18 SILAC phosphoproteomics reveals differential phosphorylation is  
19 widespread between the procyclic and bloodstream form lifecycle stages  
20 of *Trypanosoma brucei*. *J Proteome Res*, 12, 2233-44.
- 21 VANHOLLEBEKE, B., UZUREAU, P., MONTEYNE, D., PEREZ-MORGA, D. & PAYS, E.  
22 2010. Cellular and molecular remodeling of the endocytic pathway during  
23 differentiation of *Trypanosoma brucei* bloodstream forms. *Eukaryot Cell*,  
24 9, 1272-82.
- 25 VASSELLA, E., KRAMER, R., TURNER, C. M., WANKELL, M., MODES, C., VAN DEN  
26 BOGAARD, M. & BOSHART, M. 2001. Deletion of a novel protein kinase  
27 with PX and FYVE-related domains increases the rate of differentiation of  
28 *Trypanosoma brucei*. *Mol Microbiol*, 41, 33-46.
- 29 VASSELLA, E., REUNER, B., YUTZY, B. & BOSHART, M. 1997. Differentiation of  
30 African trypanosomes is controlled by a density sensing mechanism  
31 which signals cell cycle arrest via the cAMP pathway. *J Cell Sci*, 110 ( Pt  
32 21), 2661-71.
- 33 WINTER, V. & HAUSER, M. T. 2006. Exploring the ESCRTing machinery in  
34 eukaryotes. *Trends in Plant Science*, 11, 115-123.
- 35 ZAMAN, S., LIPPMAN, S. I., ZHAO, X. & BROACH, J. R. 2008. How *Saccharomyces*  
36 responds to nutrients. *Annu Rev Genet*, 42, 27-81.
- 37  
38

## Figure Legends

### Figure 1

*A schematic representation of the infection dynamics of T. brucei growth in the mammalian host.*

Upon reaching a threshold density (and SIF levels) slender forms undergo differentiation to stumpy forms, characterised by G1/G0 arrest as well as morphological changes. Thereafter, the parasitaemia declines, until the next wave emerges due to the proliferation of slender forms with a new VSG coat, this evading antibodies raised to parasites in the first peak. The stumpy forms play a crucial role in transmission, being pre-adapted for survival in the tsetse fly and differentiation to procyclic forms in the tsetse midgut. The growth arrest of stumpy forms is key to maintenance of infection chronicity, as curtailment of the parasitaemia ensures prolonged survival of the host.

### Figure 2

*In vitro induction of stumpy-like forms using 8pCPT-cAMP/AMP (a) and use of this response in a genome-wide RNAi screen to identify components of the stumpy induction pathway (b).*

(a) Pleomorphic parasites are capable of responding to SIF, giving rise to stumpy forms. However, monomorphs are non-responsive to SIF but, instead, are capable of being induced to stumpy-like forms by cell permeable, hydrolysable, cAMP/AMP analogues. (b) A monomorphic RNAi library was exposed to 8pCPT cAMP/AMP. Those parasites that had a gene required in the cAMP/AMP response pathway depleted (red), remained slender and continued proliferating, while the others (green) underwent growth arrest. The resistant parasites eventually outgrew and predominated the population. DNA was extracted from the selected cells, the RNAi inserts amplified by PCR and then subjected to ion-torrent deep sequencing to identify the genes (A and B) involved in relaying the 8pCPT-cAMP/AMP signal. The identified

genes were then validated through individual RNAi lines in pleomorphs to confirm their role in physiological SIF signalling (adapted from (Mony et al., 2013)).

### Figure 3

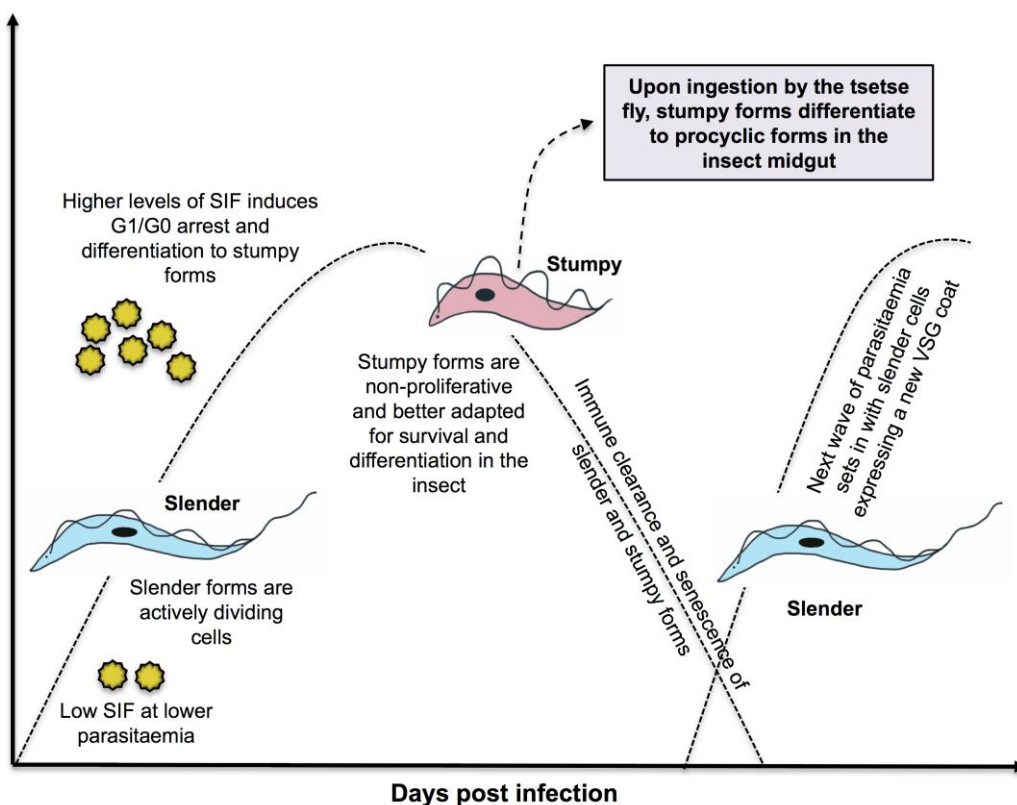
*The balance between 'slender retainers' (SR) and 'stumpy inducers' (SI) controls stumpy formation.*

The slender cells remain proliferating as long as the levels of SR are high and SI are low. However, upon an increase in cell density, SIF accumulates, triggering a quorum-sensing like response that induces the activation of SIs with a concomitant repression of the SRs. It is the combined action of 'switching-off' of the slender retention (SR) arm and 'switching-on' of the stumpy induction (SI) arm that ultimately drives the formation of stumpy cells

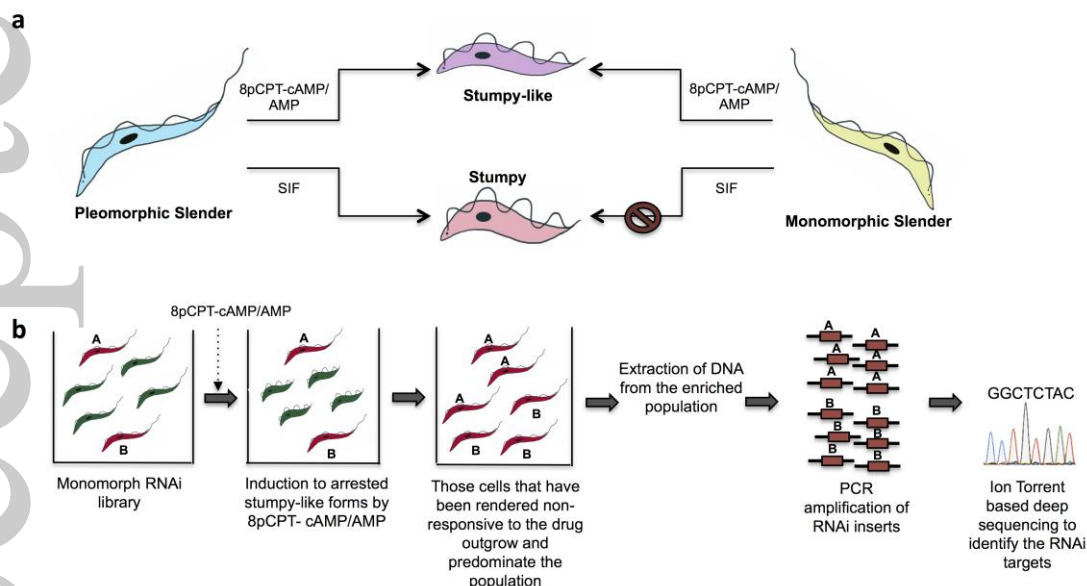
### Figure 4

*A hypothetical framework for the molecular control of stumpy formation.*

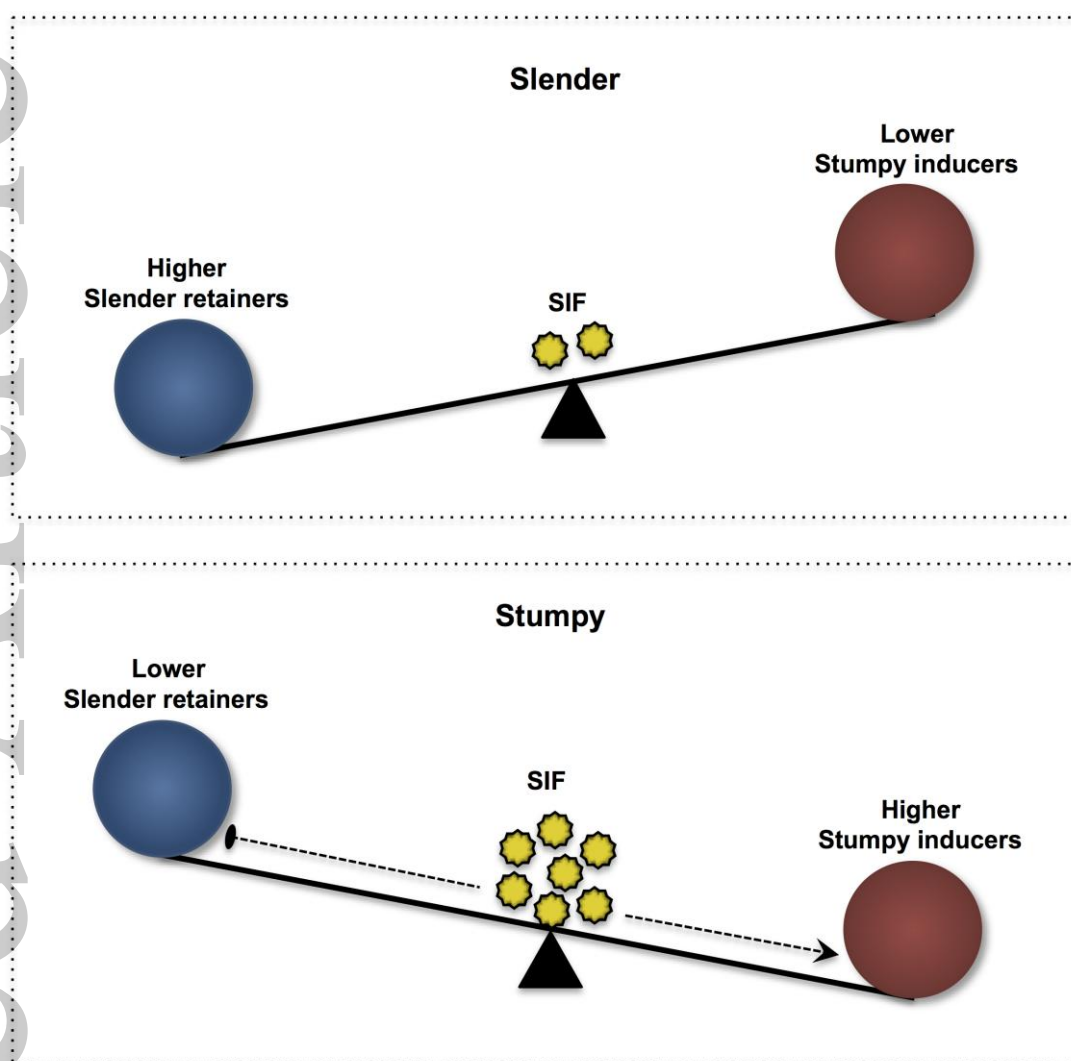
The figure shows an integration of all known networks linked to starvation responses and genes identified in the genome-wide RNAi screen for drivers of stumpy formation. Many of the components included remain to be validated in independent RNAi or knock out lines and so their inclusion is speculative.



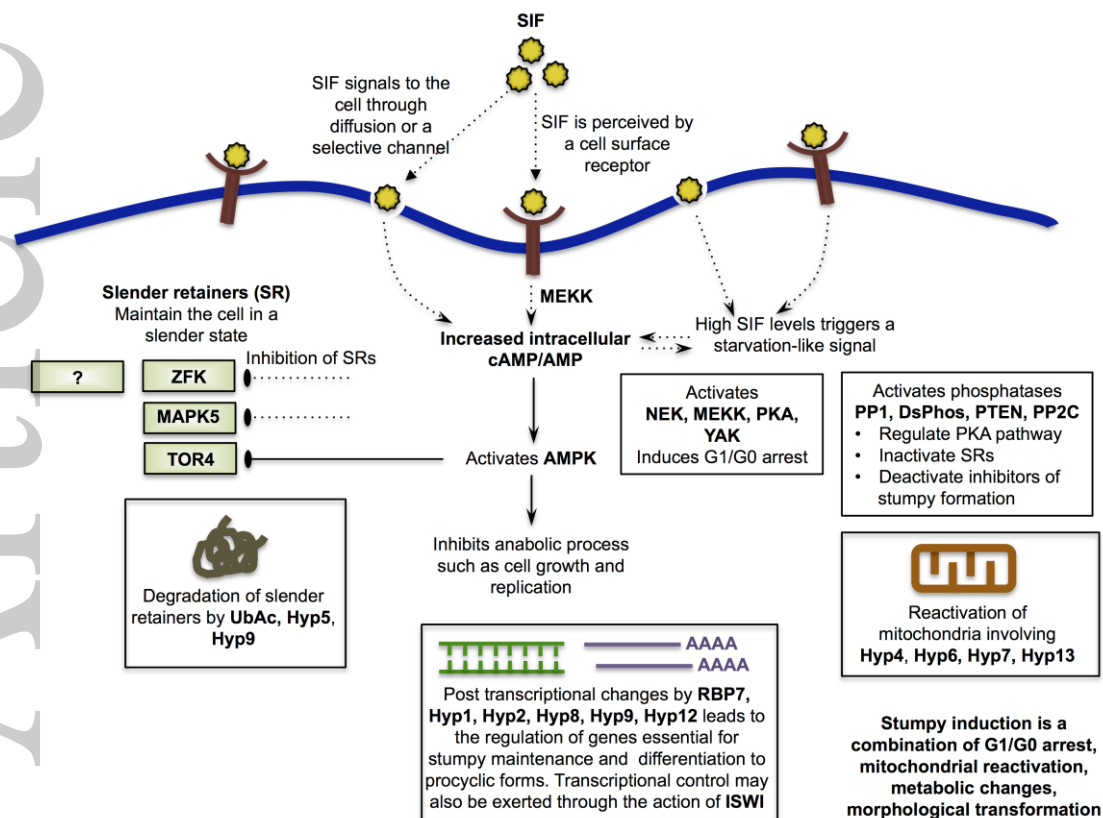
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